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Homodimerization of Neuropeptide Y Receptors Investigated by Fluorescence Resonance Energy Transfer in Living Cells*

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Up to now neuropeptide Y (NPY) receptors, which belong to the large family of G-protein-coupled receptors and are involved in a broad range of physiological processes, are believed to act as monomers. Studies with the Y_1-receptor antagonist and Y_4-receptor agonist GR231118, which binds with a 250-fold higher affinity than its monomer, led to the first speculation that NPY receptors can form homodimers. In the present work we used the fluorescence resonance energy transfer (FRET) to study homodimerization of the hY_1-, hY_2-, and hY_5-receptors in living cells. For this purpose, we generated fusion proteins of NPY receptors and green fluorescent protein or spectral variants of green fluorescent protein (cyan, yellow, and red fluorescent protein), which can be used as FRET pairs. Two different FRET techniques, fluorescence microscopy and fluorescence spectroscopy, were applied. Both techniques clearly showed that the hY_1-, hY_2-, and hY_5-NPY receptor subtypes are able to form homodimers. By using transiently transfected cells, as well as a stable cell line expressing the hY_2-GFP fusion protein, we could demonstrate that the Y-GFP fusion proteins are still functional and that dimerization varies from 26 to 44% dependent on the receptor. However, homodimerization is influenced neither by NPY nor by Gα protein binding.

G-protein-coupled receptors (GPCRs)[1] represent a superfamily of proteins characterized by seven transmembrane α-helices that interact with a family of heterotrimeric GTP-binding proteins, referred to as G-proteins (1). GPCRs are found in a wide range of organisms, and many kind of chemical messengers act through them, for example adrenalin, angiotensin, or neuropeptide Y (NPY). Ligands for GPCRs are involved in a broad range of physiological functions, and their malfunction is responsible for many diseases (2, 3).

Until recently GPCRs were thought to function as monomers. However, a growing number of evidence suggests that they may exist as homodimers and heterodimers (4–9). The existence of homodimers has been shown for several GPCRs including β_2-adrenergic receptor (10–12), δ- and κ-opioid receptors (6, 13), metabotropic glutamate receptor 5 (14), calcium-sensing receptor (15–17), m3 muscarinic receptor (18, 19), vasopressin V2-receptor (20), somatostatin (21, 22), and dopamine receptors (23–25). Whereas homodimerization of the somatostatin receptor 5 (21), the δ-opioid receptor (13), and the β_2-adrenergic receptor (11) are agonist-mediated, dimerization of the κ-opioid receptor (26) is agonist-independent.

So far photoaffinity labeling (27), cross-linking studies (15, 24), Western blot analysis (14), and immunoprecipitation (17, 28, 29) are the most frequently applied methods for the investigation of receptor homodimerization. Because of the development of new fluorescent dyes, novel fluorescent proteins, and new instrumentation, the fluorescence resonance energy transfer (FRET) obtained a renaissance (30) and could be applied recently for the investigation of receptor dimerization, as well (11, 21, 31). This technique measures the transfer of energy from a donor molecule to an acceptor molecule when they are between 1–10 nm apart (Fig. 1) (32). The FRET technique was successfully applied for the GPCR gonadotropin-releasing hormone receptor (31). Furthermore, FRET was successfully used for the investigation of transmembrane transporters like the serotonin transporter (33) and epidermal growth factor receptor (34).

In the present study, we investigate the hY_1-, hY_2-, and hY_5-NPY receptor subtypes for their ability to form homodimers by direct FRET of receptor-GFP fusion proteins. NPY receptors belong to the rhodopsin-like superfamily of G-protein-coupled receptors (35). So far five distinct NPY receptors have been cloned (Y_1, Y_2, Y_4, Y_5, and y_6) (36). Their activation results in the inhibition of adenylyl cyclase and in an increase in intracellular calcium concentration. The natural ligand of these receptors is NPY, a 36-amino acid peptide amide that belongs to the family of pancreatic polypeptides (37). NPY modulates numerous physiological processes including regulation of cardiovascular (38) and renal functions, intestinal motility, memory (39), anxiety, seizures, feeding (40), circadian rhythm (41), and nociception. First speculations for homodimerization come from studies with recently reported truncated NPY analogues named ([P^{30},C^{31},F^{32},L^{34}] NPY28–36 and [P^{30},C^{31},W^{32},L^{34}]NPY28–36) (42, 43) that bind after dimerization with a higher affinity to the hY_1-receptor. Furthermore, the homodimeric, peptidergic GR231118 (Y_1-antagonist, Y_4-agonist) obtains a higher affinity for the Y_1-receptor than its monomeric form (42, 43).

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This paper is dedicated to Professor Günther Jung, University of Tübingen, on the occasion of his 65th birthday.

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[1] The abbreviations used are: GPCR, G-protein-coupled receptor; BHK, baby hamster kidney; CFP, cyan fluorescent protein; CHO, Chinese hamster ovary; DSRed, red fluorescent protein; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; NPY, neuropeptide Y; YFP, yellow fluorescent protein; EGFP, enhanced GFP; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; FRET^c, corrected FRET; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; wt, wild-type.

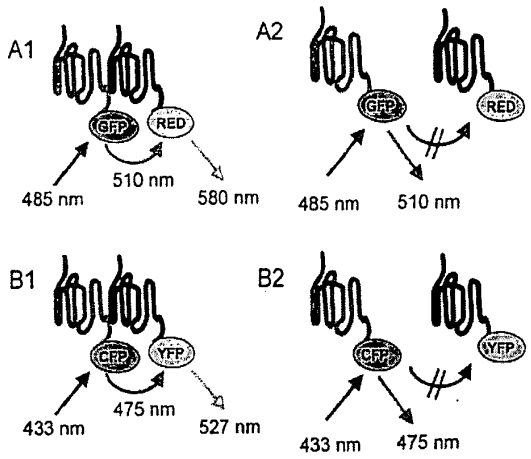


FIG. 1. **Schematic diagram of FRET.** Energy transfer from the donor (*GFP, CFP*) to the acceptor molecule (*DSRed, YFP*) is only possible when the distance is lower than 100 Å (*A1, B1*). Otherwise, FRET is not measurable (*A2, B2*).

To investigate homodimerization of NPY receptors by FRET, we generated fusion proteins of the hY_1-, hY_2-, or hY_5-receptor sequence tagged at their carboxyl terminus to the green, cyan, yellow, or red fluorescent protein (GFP, CFP, YFP, DSRed), respectively. The advantage of these proteins is that they have the suited properties to be used as FRET pairs (44–46). CFP and YFP are spectral variants of GFP and have an appropriate spectral overlap of the donor (CFP) emission and the acceptor (YFP) excitation (47). This pair of proteins has been used widely for the investigation of protein-protein interaction (33, 34). The FRET pair with GFP as donor and DSRed as acceptor is a second successfully applied FRET pair (31). Binding and functional studies showed that these receptor-GFP fusion proteins are still active. Furthermore, the establishment of a cell line that stably expresses the hY_2-GFP fusion protein was generated to quantify receptor expression and to correlate the number of binding sites to the fluorescence emission. Coexpression of the FRET pair receptors hY_1-CFP/hY_1-YFP, hY_1-GFP/hY_1-DSRed, hY_2-CFP/hY_2-YFP, hY_2-GFP/hY_2-DSRed, hY_5-CFP/hY_5-YFP, and hY_5-GFP/hY_5-DSRed was analyzed by fluorescence microscopy and fluorescence spectroscopy. A significant FRET effect was found with both techniques and both FRET pairs in a receptor subtype-dependent manner. Whereas the hY_2-receptor was less prone to dimerization, hY_1 and hY_5 had a strong tendency. However, the FRET effect was neither dependent on agonist stimulation nor on GTPγS incubation as shown by quantitative fluorescence spectroscopy.

EXPERIMENTAL PROCEDURES

Materials

All materials for tissue culture were supplied by Invitrogen. ^{3}H-NPY was purchased from Amersham Biosciences (specific activity 2.59 TBq/mmol). Restriction enzymes and modifying enzymes were from Invitrogen, and forskolin, Pefabloc SC, pepstatin, *N*-(*trans*-epoxysuccinyl)-L-leucine-4-guanidinobutylamide (E64), sodium chloride, magnesium sulfate, potassium chloride, 3-isobutyl-1-methylxanthine, and glucose were from Fluka (Taufkirchen, Germany). Bovine serum albumin, bacitracin, puromycin, and HEPES were supplied by Sigma. Oligonucleotides were purchased from MWG-Biotech AG (München, Germany).

Vector Construction

To create human NPY Y_1-, Y_2-, Y_5-receptor fusion proteins with GFP, CFP, YFP, and DSRed, respectively, appropriate expression vectors were cloned. PCR with *Taq* polymerase was used to amplify the hY_1-, hY_2-, and hY_5-receptor sequences, which are cloned in the pcDNA3 vector (Invitrogen). An amino-terminal primer, 5′-CTGCTTACTGGCTATCG-3′, upstream of the *Hin*dIII site of the multicloning site of pcDNA3 was combined with a primer designed against the carboxyl terminus, where the stop codon is replaced by a restriction site and there is an additional codon for alanine. The carboxy-terminal primer, 5′-CGCGGATCCCAGATTTTTTCATTATCATCATTGTTG-3′, with a *Bam*HI site, was used for amplifying the hY_1-sequence, the carboxy-terminal primer, 5′-CGCGGATCCGCGACATTGGTAGCCTCTG-3′, with a *Bam*HI site, was used for amplifying the hY_2-sequence, and the antisense primer, 5′-CGCGTCGACCCCATATGAAGACAGTGTATAA-G-3′, with a *Sal*I site, was used for amplifying the hY_5-sequence. After PCR the receptor sequences were subcloned into pCR2.1-TOPO vector (Invitrogen), respectively, amplified, and digested with *Hin*dIII and *Bam*HI or *Hin*dIII and *Sal*I. To obtain the hY_1-pEGFP, hY_2-pEGFP, and hY_5-pEGFP vectors the plasmid pEGFP-N1 (Clontech) was digested either with *Hin*dIII and *Bam*HI or with *Hin*dIII and *Sal*I. The digested vector was ligated to the receptor sequences obtained from pCR2.1-TOPO vector, respectively. Expression vectors with the hY_1-, hY_2-, and hY_5-sequence and CFP, YFP, and DSRed, respectively, were cloned in the same way by using the pECFP-N1 vector (Clontech), pEYFP-N1 vector (Clontech), or the pDSRed-N1 vector (Clontech). The constructs were verified by restriction and sequence analysis.

Construction of Positive Control Fusion Proteins

As a positive control for FRET imaging and measurements, we constructed two different fusion proteins of hY_1-receptor, GFP and DSRed and hY_2-receptor, YFP and CFP. The hY_1-GFP-DSRed vector was cloned in two steps. First, a hY_1-pEGFP fusion protein without stop codon in the fusion protein sequence was generated. Therefore, the hY_1-pEGFP vector was used as template, and PCR was performed with the amino-terminal primer, 5′-ATCTGGGATCCACCGG-3′, and the carboxy-terminal primer, 5′-TCCTCCGCGGCCGCTCCCTTGTACAG-CTCG-3′, to generate the hY_1-$GFP^{(-stop)}$ sequence. After digestion the hY_1-GFP sequence of the hY_1-pEGFP vector was replaced by hY_1-$GFP^{(-stop)}$. In the second step the DSRed sequence was amplified by using PCR. Two primers were generated, the sense primer, 5′-GGCG-GCGGCGCGGCCGCGATCAATCTCATCACCAGACAGAGATATGGC-AAGAGATCCAGCCCTGAGACACTGATTGGCATGGTGCGCTCCTC-C-3′, with a *Not*I site and a restriction site for convertase I, and the antisense primer, 5′-GCCTCTAGAGCCCTACAGGAACAGGTGG-3′, with a *Xba*I site. The vector hY_1-$pEGFP^{(-stop)}$ was digested with *Bam*HI and *Xba*I, and the DSRed sequence was subcloned. The construct was verified by restriction and sequence analysis.

For the cloning of the hY_2-YFP-CFP vector the pEYFP-CFP-tandem vector, which was a generous gift from Professor Horn (University of Leipzig), was used as backbone, and the hY_2-receptor sequence was amplified by PCR. The amino-terminal primer, 5′-GGCGGCGCTAG-CATGGGTCCAATAGGTGC-3′, with a *Nhe*I site, and carboxy-terminal primer, 5′-GGCGGCCTCGAGGGCGACATTGGTAGCCTCTG-3′, with a *Xho*I site, were used for PCR. The hY_2-CFP-YFP-tandem was digested with *Nhe*I and *Xho*I, and the hY_2-sequence was subcloned. The vector was verified by restriction and sequence analysis.

Cell Culture and Transfection

BHK cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, and CHO cells were grown in 50% Dulbecco's modified Eagle's medium/50% nutrient mix Ham's F-12 with 10% fetal calf serum. The cells were cultured as monolayers in a humidified 5% CO_2 atmosphere at 37 °C. BHK and CHO cells do not express endogenous NPY receptors.

For transient transfection cells were seeded in 25-cm^2 flasks. At 90% confluency each well was transiently transfected with 13 μg of reporter plasmid DNA using 26 μl of LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. For cotransfection 6.5 μg of each vector was used. For transient transfection 4.7 μg of vector and 9.4 μl of LipofectAMINE 2000 was used for each well of a six-well plate, and 1 μg of vector and 2 μl of LipofectAMINE 2000 was applied for each well of a 24-well plate.

To generate a cell line stably expressing hY_2-GFP receptors BHK cells were cotransfected with hY_2-GFP and pBabePuro vector (48). Three days after transfection cells were seeded and maintained in medium with 8 μg/ml puromycin. The medium was changed every 4 days. After 4 weeks stable transformants were isolated by FACS sorting with a Vantage SE FACS machine (BD Biosciences). FACS sorting was repeated three times. There was a period of 4 weeks between each sorting. Clonal expression was confirmed by fluorescent microscopy.

Competition Binding Assay

For binding studies BHK or CHO cells were transiently transfected with the corresponding plasmid 24 h before analysis. Cells were resuspended in incubation buffer (minimum essential medium with Earl's salts containing 0.1% bacitracin, 50 mM Pefabloc SC, and 1% bovine serum albumin). 200 μl of the suspension, containing 4.0×10^{-6} cells per ml, were incubated with 25 μl of a 10 nM solution of ^{3}H-propionyl-NPY (specific activity 2.59 TBq/mmol) and 25 μl of NPY in a concentration range of 1 pM to 1 μM. Nonspecific binding was defined in the presence of 1 μM unlabeled NPY. After 1.5 h at room temperature, the incubation was terminated by centrifugation at 1600 × *g* at 4 °C for 5 min. The pellets were washed once with 400 μl of cold PBS and centrifuged again, and the washed pellets were resuspended in 100 μl of PBS. The cell suspension was mixed with 3 ml of scintillation mixture, and radioactivity was measured by a β-counter. The IC_{50} value was calculated with the software Prism 3.0 (Graph Pad). Each experiment was performed in triplicate, and IC_{50} ± S.E. are shown in Table I.

For saturation experiments BHK cells stably expressing hY_2-GFP were incubated with increasing concentrations of ^{3}H-propionyl-NPY between 0.1 pmol and 8 nmol. Nonspecific binding was determined in the presence of 10^{-6} M unlabeled porcine NPY. The assay was performed three times independently.

Assays for cAMP Concentration

The intracellular amount of cAMP was determined either by using a cAMP enzyme immunoassay (Biotrak, Amersham, Freiburg, Germany) or with a luciferase reporter gene assay. To perform cAMP enzyme immunoassays BHK or CHO cells were transiently transfected with the corresponding plasmid 24 h in advance. When the cells were grown to confluency, they were resuspended in cAMP buffer (145 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl, 10 mM HEPES, 0.5% bovine serum albumin, 10 mM glucose, 0.1 mM 3-isobutyl-1-methylxanthine, pH 7.4, and incubated for 10 min at 37 °C. Then 2 million cells were incubated with 15 μM forskolin and 1 μM NPY for 1 h at 37 °C. Cells were preincubated with 1 μM NPY for 1 h and then forskolin was added. Incubation was stopped by addition of 100 μl of a 1 M solution of HCl. Cell lysis was done by freezing, followed by centrifugation, and the supernatant was diluted 1:5. Reactions were performed according to the protocol of the manufacturer for the cAMP enzyme immunoassay. The optical density was determined with a plate reader (Tecan SpectraFluorPlus) at 450 nm. The assay was done in duplicate and repeated three times. The reporter gene assay using the pCRELuc plasmid (Stratagene) was performed as reported recently (49).

Fluorescence Microscopy

Fluorescence microscopy with cells coexpressing CFP and YFP receptor fusion proteins was done on a Leica DM IRB microscope (Leica, Wetzlar, Germany). BHK cells were seeded on 24-well plates and transiently cotransfected with the corresponding constructs 24 h before studying by fluorescence microscopy. As excitation source a polychrome VI laser from Photonics was used. CFP was excited at 433 nm, and YFP was excited at 513 nm, and emission was detected with different filter sets (AHF Analysentechnik, Tübingen, Germany). A CFP filter with a D480/40 emitter and a 455 DCLP beamsplitter, a YFP filter with an HQ535/30 emitter and a Q515 lp beamsplitter and a FRET filter (emitter D535/30, beamsplitter 455 DCLP) were used. For FRET microscopy studies cells were excited at 433 nm, and for emission detection the YFP filter was used. Cells were washed two times with PBS and covered with 0.5 ml of PBS and then images were taken with a cooled digital Orca-ER camera (Hamamatsu, Herrsching, Germany). For imaging the openlab software (Improvision, Heidelberg, Germany) was used.

For quantitative FRET measurement the method of sensitized FRET was used (34). Calculations of corrected FRET ($FRET^c$) were carried out on a pixel-by-pixel basis for the entire image. The bleedthrough of CFP and YFP through the FRET filter channel was regarded exerting the following equation: $FRET^c$ = FRET − (bleedthrough CFP × CFP) − (bleedthrough YFP × YFP). $FRET^c$ images are displayed as YFP intensity-modulated images (51) in pseudocolor mode, where red areas display high values of FRET, and blue areas display low values of FRET. The overall intensity of FRET was calculated using openlab software.

Confocal Imaging

General—BHK cells expressing both NPY receptor-C tail-GFP and NPY receptor-C tail-DSRed were imaged in a 6-well plate in PBS at room temperature in a Zeiss LSM 510 confocal microscope using a water immersion objective. GFP was excited with the 488-nm line of an argon laser, and DSRed was excited with the 568-nm line of a krypton laser. Emission was measured simultaneously in the green channel and in the red channel.

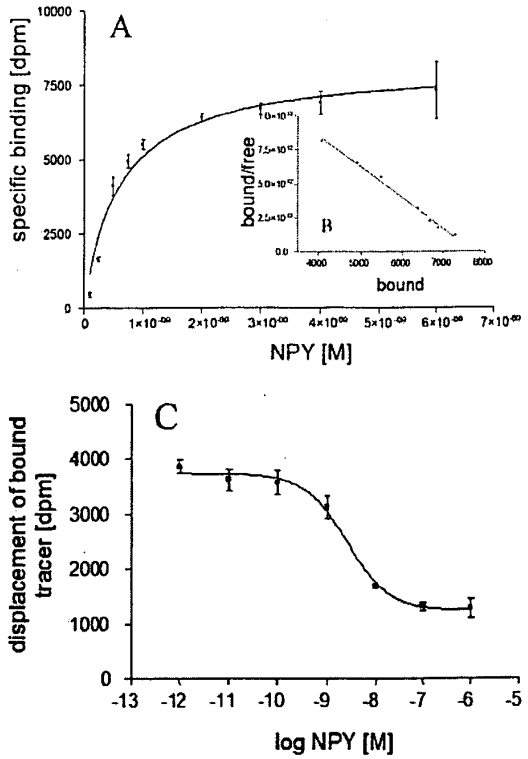


FIG. 2. **Characterization of BHK cells stably expressing the hY_2-GFP fusion protein.** *A*, saturation curve; *B*, Scatchard analysis. A K_d value of 0.45 ± 0.15 nM and 7063 ± 764 binding sites/cell were calculated. An IC_{50} value of 2.6 ± 1.3 nM for NPY was determined (*C*).

Photobleaching FRET Analysis—FRET was measured in fixed cells by continuous illumination of the acceptor (red fusion protein) with the 568-nm line of the Kr laser, which was sufficient to bleach the acceptor. Images of GFP were taken before and after the red fluorescent protein was photobleached. The increase of donor fluorescence (green) after receptor (red) bleaching was interpreted as evidence of FRET occurring from GFP to DSRed.

Fluorescence Spectroscopy

The spectrofluorometric studies were carried out at a Fluorolog-3 spectrofluorometer (Jobin Yvon Spex, Longjumeau, France). For excitation a 450-watt xenon lamp was used.

To study FRET effects between the different NPY receptor subtypes BHK cells were seeded in 25-cm^2 culture flasks. The cells were transiently cotransfected with equal amount of the same receptor subtype fused to CFP and YFP, respectively, 24 h before measurements.

The cells were washed with PBS, treated with trypsin/EDTA, and resuspended with Dulbecco's modified Eagle's medium containing fetal calf serum and protease inhibitors (Pefabloc 5 mM, pepstatin 0.7 μg/ml, E64 10 μg/ml). For each measurement 1.0×10^6 cotransfected cells from the same cell pool were incubated with 1 μM, 0.1 μM, and 0.1 nM NPY for 30 min, respectively, and in parallel, the cells were also incubated with 1 μM NPY for 10, 30, and 60 min. To investigate the effect of GTPγS cells were incubated with 50, 100, or 500 μM GTPγS in the presence of 1 μM NPY for 30 min. After incubation cells were centrifuged at 160 × *g* at room temperature for 5 min. Then the cell pellets were washed two times with 0.5 ml of PBS and for measurements resuspended in 1 ml of PBS containing protease inhibitors (Pefabloc 5 mM, pepstatin 0.7 μg/ml, E64 10 μg/ml).

For CFP and FRET measurements emission wavelength scans were performed from 450–560 nm with an excitation wavelength of 433 nm. YFP scans were recorded from 510–570 nm with an excitation wavelength of 488 nm.

For the negative control NPY receptor fusion proteins with CFP were cotransfected with the pEYFP-N1 vector, and emission scans were

TABLE I
NPY binding properties of fusion proteins

Receptor	Binding assay			
	IC_{50}	Total binding	Unspecific binding	Specific binding
	nM			
$hY_{1(wt)}$	1.6 ± 0.5	2957 ± 71	1095 ± 44	1862 ± 96
hY_1-GFP	2.6 ± 0.5	4481 ± 109	719 ± 163	3762 ± 226
hY_1-DSRed	3.0 ± 0.9	3521 ± 245	957 ± 171	2561 ± 345
hY_1-CFP	1.6 ± 1.1	2958 ± 71	1095 ± 44	1862 ± 96
hY_1-YFP	10.2 ± 0.4	7379 ± 1161	819 ± 103	6559 ± 1346
$hY_{2(wt)}$	5.4 ± 1.1	1594 ± 21	1185 ± 5	410 ± 25
hY_2-GFP	13.0 ± 4.0	2958 ± 71	1095 ± 44	1862 ± 96
hY_2-DSRed	8.6 ± 0.7	2016 ± 50	1037 ± 93	979 ± 122
hY_2-CFP	3.8 ± 1.4	6703 ± 135	1510 ± 16	5193 ± 157
hY_2-YFP	2.1 ± 0.5	6295 ± 58	1326 ± 8	4969 ± 68
$hY_{5(wt)}$	7.2 ± 5.0	1895 ± 262	1173 ± 51	817 ± 340
hY_5-GFP	16.1 ± 5.0	6820 ± 360	1237 ± 54	5583 ± 420
hY_5-DSRed	16.2 ± 9.2	3308 ± 244	1065 ± 104	2242 ± 306
hY_5-CFP	5.6 ± 2.3	2958 ± 71	1095 ± 44	1862 ± 96
hY_5-YFP	6.8 ± 3.5	5083 ± 699	692 ± 280	4391 ± 869
hY_1-GFP-DSRed	3.1 ± 2.0	2816 ± 178	1174 ± 478	1641 ± 589
hY_2-YFP-CFP	1.8 ± 0.1	5400 ± 5.87	1478 ± 40	3922 ± 47

performed at 433- and 488-nm excitation. For transfection 1 μg of the pEYFP vector and 6.5 μg of CFP fusion protein vector were used, which resulted in a higher expression of YFP.

Fluorescence derived from non-transfected cells and equipment was subtracted from each sample to obtain specific fluorescence. The emission spectra at 433-nm excitation were normalized to the CFP fluorescence peak at 475 nm.

RESULTS

Vector Construction

For the investigation of NPY receptor homodimerization by FRET technique different fusion constructs of the hY_1-, hY_2-, and hY_5-receptor and GFP, CFP, YFP, and DSRed, respectively, were cloned. To obtain fusion proteins PCR was used to amplify the receptor sequences. The receptor sequences were subcloned into the expression vectors with GFP, CFP, YFP, and DSRed, respectively. The primers were generated by first removing the stop codon of the receptor sequences and second including restriction sites that allow an in-frame cloning. The cloning was proven by restriction analysis and sequencing. The sequencing data showed that there was no mutation for the hY_1-receptor. For the hY_2-receptor we could find three mutations (two silent at position Ile-195 and Ile-312 and one conservative mutation K206T). Three mutations were found, as well, for hY_5-receptor (one silent at position Asp-31 and two mutations I143T and the consecutive R286K). All mutants, however, were fully functional. Expression and plasma membrane localization of the fusion proteins was confirmed by confocal laser scanning microscopy (see Fig. 3*A*).

Characterization of BHK Cells Stably Expressing hY_2-GFP Fusion Protein

To establish a cell line that stably expresses the hY_2-GFP fusion protein, BHK cells were transfected with hY_2-pEGFP vector and cultivated in selection medium. Positive clones were separated by using preparative FACS techniques. Cells expressing the NPY receptors were tested by using radioligand binding assay, and GFP expression was investigated by fluorescence microscopy. An IC_{50} value of 2.6 ± 1.3 was determined by using ^{3}H-propionyl-NPY and unlabeled NPY in a competition assay (Fig. 2*C*). For determination of the K_d value saturation experiments were performed. A typical saturation curve and Scatchard analysis of ^{3}H-propionyl-NPY binding to BHK cells stably expressing the hY_2-receptor fusion protein (hY_2-GFP/BHK) is shown in Fig. 2. K_d value of 0.45 ± 0.15 nM and 7063 ± 764 binding sites/cell were calculated after Scatchard analysis.

Functionality of NPY Receptor Fusion Constructs

To prove whether the fusion proteins are still functional competition assays for binding and cAMP assays as functional assay were performed. The competition assays showed that all fusion proteins can still bind the natural ligand NPY. The IC_{50} values of all receptor fusion proteins are shown in Table I, and these values are comparable with IC_{50} values from binding studies with the wild type (wt) receptors. For the $hY_{1(wt)}$-receptor an IC_{50} of 1.6 ± 0.5 nM, for the $hY_{2(wt)}$-receptor an IC_{50} of 5.4 ± 1.1 nM, and for the $hY_{5(wt)}$-receptor an IC_{50} of 7.22 ± 5.0 nM was determined.

The cAMP assays confirmed that all fusion proteins are still active and still inhibit adenylyl cyclase. NPY receptors are G_i-coupled receptors and act through inhibition of adenylyl cyclase. This was measured by the inhibition of forskolin-stimulated cAMP accumulation after receptor activation. Forskolin itself is a natural activator of the adenylyl cyclase. The inhibition of the adenylyl cyclase was found for all receptor fusion proteins after incubation with NPY (data not shown). This confirms that the receptors can still interact with the G_i-proteins and subsequently inhibit the adenylyl cyclase.

Fluorescence Microscopy

Investigation of Coexpressed NPY Receptor Fusion Proteins—BHK cells were transiently cotransfected with the FRET pairs CFP/YFP and GFP/DSRed fused to the NPY receptor subtypes, respectively. If molecules of GFP/DSRed and CFP/YFP come into appropriate physical proximity, energy transfer from CFP to YFP and GFP to DSRed will take place (Fig. 1), and yellow or red fluorescence will be visible. Accordingly, if receptors are present as individual monomers, their fluorescence will not be effected by each other.

Fluorescence microscopy was used to perform FRET studies in living cells. Images were taken from cells cotransfected with NPY receptors fused to the FRET pair CFP/YFP. Using the corresponding donor and acceptor filter sets CFP and YFP are visible (Fig. 3*B*). Furthermore the expression of this FRET pair is not interfering with each other. To investigate NPY receptor interaction images were taken with the FRET filter set. A weak yellow fluorescence was obtained from cells cotransfected with hY_1-, hY_2-, and hY_5-receptors fused to CFP and YFP, respectively (Fig. 3*B*). The fluorescence images taken with the FRET

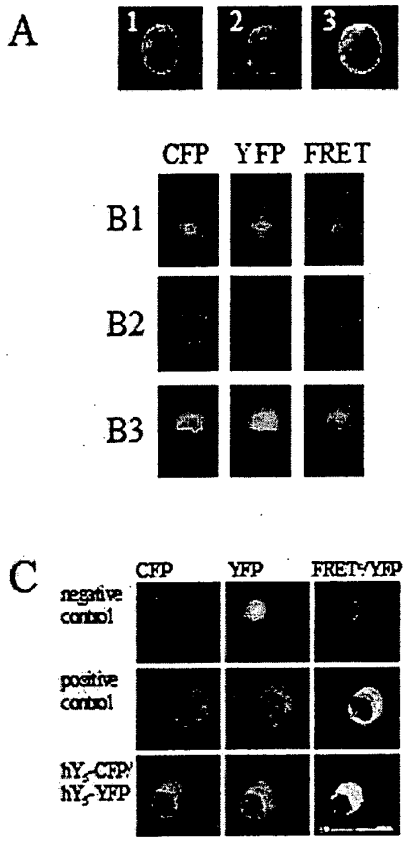


FIG. 3. **Fluorescence microscopy studies.** *A*, BHK cells were transiently cotransfected with hY_1-GFP and hY_1-DSRed fusion proteins, and images were acquired through GFP (*A1*) and DSRed (*A2*) channels using confocal laser scanning microscopy. *A3* represents an overlay of the GFP and DSRed channel. *B*, BHK cells were transiently cotransfected with equal amounts of the same NPY receptor subtype fused to the FRET pair CFP/YFP. One day after transfection, cells were washed with PBS, and images were taken with appropriate filter sets. *B1* images show hY_1-CFP/hY_1-YFP, *B2* images show hY_2-CFP/hY_2-YFP, and *B3* images show hY_5-CFP/hY_5-YFP cotransfected cells. Images were taken with the donor filter set (*left column*), with the acceptor filter set (*middle column*), and with the FRET filter set (*right column*). *C*, hY_5-CFP and hY_5-YFP receptor fusion proteins were transiently coexpressed in BHK cells. Images were taken by the CFP filter (*left column*), YFP filter (*middle column*), and the FRET filter. $FRET^c$ was calculated as described under "Experimental Procedures" and is presented as pseudocolor intensity-modulated images ($FRET^c$/YFP; *right column*).

filter also showed that the fluorescence intensity of the hY_1- and hY_5-receptor-transfected cells was stronger than the fluorescence from hY_2-receptor-transfected cells, whereas the conditions were equal for all systems.

We suggest that the weak yellow fluorescence is an effect of FRET and results from a close contact between the receptor subtypes. For negative controls, cells expressing only YFP or CFP fusion proteins were investigated with the appropriate FRET filter, as well. We observed no yellow fluorescence by using the same FRET filter and the same conditions as above (Fig. 4, *A* and *B*).

Furthermore, for positive control BHK cells were transfected with the hY_2-YFP-CFP fusion protein, and fluorescence images were taken with the corresponding filter set. The expression of the FRET partners was not disturbed, and yellow and cyan fluorescence was obtained. By taking the FRET filters a yellow fluorescence was observed (Fig. 4*D*, comparable with the one in Fig. 3*B*).

Effect of NPY—To study NPY-induced FRET effect BHK cells were cotransfected with hY_1-GFP and hY_1-DSRed fusion protein. Cells were covered with medium containing 1 μM NPY and incubated for 30 min. Images were taken and compared with images from non-stimulated cells. No increase or decrease of fluorescence intensity was observed (data not shown).

Quantitative FRET Measurement—To quantify FRET the efficiency of energy transfer between CFP and YFP was measured by calculating the sensitized FRET signal ($FRET^c$) on a pixel-by-pixel basis as described under "Experimental Procedures" (50). A strong energy transfer was found for the positive control, and the lowest energy transfer was found for the negative control. A strong energy transfer is illustrated with the red color, and a low intensity is illustrated with the blue color. Furthermore the green color of NPY receptor cotransfected cells indicates that there is an energy transfer.

Photobleaching Studies—The method of photobleaching is a sufficient application for the investigation of FRET (21). FRET can occur only in the presence of an acceptor molecule. If the acceptor is removed or destroyed, it would be predicted that the fluorescence of the donor would increase upon illumination. For photobleaching studies the FRET pair GFP (donor) and DSRed (acceptor) fused to the Y_5-receptor, respectively, was used. After a strong illumination of DSRed we observed an decrease of red fluorescence and 30% increase of the green fluorescence.

Fluorescence Spectroscopy

Investigation of NPY Receptor Fusion Proteins—To quantify the FRET effect and to verify the results obtained from fluorescence microscopy FRET studies were performed with a spectrofluorometer in living cells. For measurements the cells were cotransfected with the same NPY receptor subtype fused to CFP and YFP, respectively. Under these conditions FRET should be observed if CFP and YFP were in close proximity.

Cells cotransfected with the hY_5-receptor fusion proteins were excited at 433 nm, and emission scans were performed. The spectra showed a shoulder at 475 and 505 nm, the emission shoulder of CFP. A third significant peak was found at 527 nm, the emission maximum of YFP (Fig. 5, *A1*, *blue line* and *B*). The emission scan of cells only expressing the hY_5-CFP receptor fusion protein showed only the emission shoulder at 475 and 505 nm; the YFP emission peak at 527 nm was not observed (Fig. 5, *A1*, *brown line* and *B*). Emission scans were also performed with cells coexpressing the hY_1- and the hY_2-receptor fusion proteins, respectively. For all receptor subtypes the emission shoulder at 475 and 505 nm was recorded, as was the significant peak at 527 nm (Fig. 5*B*). In parallel emission scans were performed with cells expressing the hY_1- and hY_2-receptor CFP fusion protein, respectively, and the obtained spectra show only the emission shoulder at 475 and 505 nm (Fig. 5*B*). For the hY_1- and the hY_5-receptor fusion proteins a stronger peak at 527 nm was obtained after normalization of the spectra to the emission maxima at 475 nm than for the hY_2-receptor (Fig. 5, *A*, *red arrows* and *B*).

For negative control cells were cotransfected with the hY_1-, hY_2-, or the hY_5-receptor fused to CFP and with untagged YFP, respectively. YFP in this case is only expressed in the cytoplasm, and therefore, no strong interaction between the receptor fusion protein and YFP is expected. Recorded emission wavelength scans with excitation at 433 nm showed that there is no significant peak at 527 nm (Fig. 5, *A*, *green lines* and *B*).

The emission spectrum of the positive control, hY_2-YFP-CFP-tandem, at 433-nm excitation shows the typically shoul-

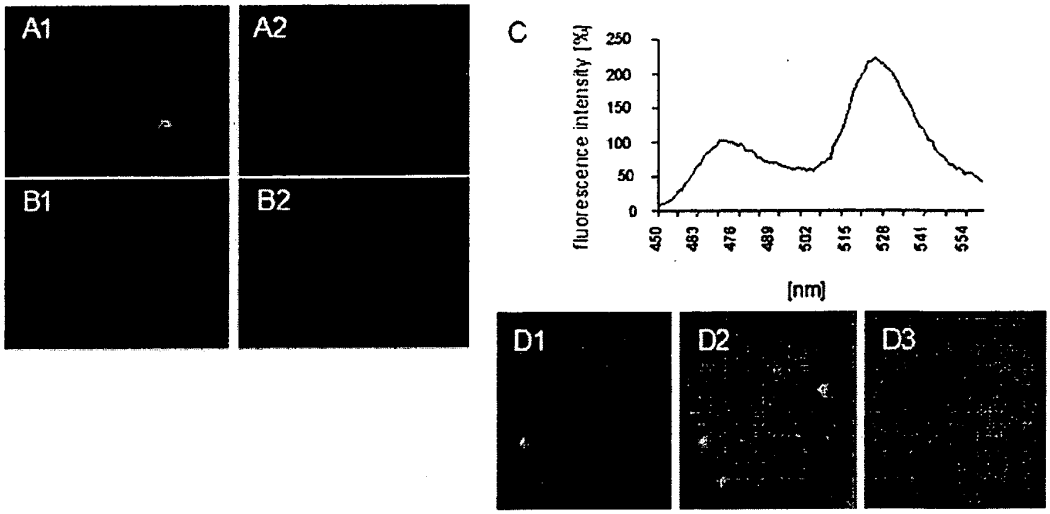


FIG. 4. **Negative and positive controls.** BHK cells were transiently transfected with hY_1-CFP (*A1*) and hY_1-YFP (*B1*), respectively, and images were taken with the corresponding filter sets. The same cells were used for imaging with the corresponding FRET filter (*A2-B2*), and no yellow fluorescence is visible. Similar studies were also performed for hY_2- and hY_5-fusion proteins and showed the same results (data not shown). As positive control BHK cells were transiently transfected with the hY_2-YFP-CFP-tandem (*D*). Images were taken with the corresponding filter sets (*D1/2*), and FRET was visible as yellow fluorescence (*D3*). The hY_2-YFP-CFP-tandem was used for fluorescence spectroscopy, and an emission scan with 433-nm excitation was performed (*C*).

der at 475 and 505 nm, obtained from CFP, and a strong peak at 527 nm (Fig. 4*C*). Because of the distance of YFP and CFP this peak can only result from FRET and is considered to be the maximal FRET.

Taken together, hY_1-, hY_2-, and hY_5-receptor fusion proteins show a significant FRET-derived increase at 527 nm, indicating that the receptors form dimers or heterodimers. The strongest fluorescence intensity at 527 nm was observed in cells expressing the hY_1- and the hY_5-receptor fusion proteins, respectively. This effect was not derived from different receptor expression as confirmed from emission spectra (Fig. 5, *A4*, *A5*, and *A6*).

Effect of the Natural Ligand NPY—To investigate a possible ligand-induced increase or decrease of fluorescence intensity at 527 nm (FRET) after excitation at 433 nm cotransfected cells were stimulated with NPY by using different conditions.

First, concentration-dependent-induced fluorescence intensity was investigated. BHK cells were cotransfected with the hY_5-CFP and hY_5-YFP fusion protein and incubated with different NPY concentrations for the same incubation time. Only cells from the same cotransfected cell pool were used for the measurements. Nearly the same fluorescence intensity at 527-nm emission was obtained for the different NPY concentrations (1 μM, 10 nM, and 0.1 nM) (Fig. 5, *A1* and *B*). The small differences of the spectra are caused by transfected cells, because each cell does not express exactly the same amount of receptor fusion proteins. Furthermore, cells cotransfected with the hY_5-receptor fusion proteins were incubated with 1 μM NPY for different incubation times (10, 30, and 60 min). Performed emission scans with excitation at 433 nm show again no difference in the emission spectra (Fig. 5, *A2* and *B*). No increase or decrease of FRET fluorescence intensity at 527 nm was observed. However, the fluorescence intensity was still significantly stronger than the fluorescence intensity of the negative control. The same approaches were performed for BHK cells coexpressing the hY_1- and the hY_2-receptor fusion proteins, respectively. No differences in the recorded spectra were found for hY_1-receptor fusion proteins. There was no increase or decrease of the FRET fluorescence intensity at 527 nm (Fig. 5*B*). The same was found for cells cotransfected with the hY_2-fusion proteins (Fig. 5*B*). Accordingly, the oligomerization of the receptor is not effected by NPY.

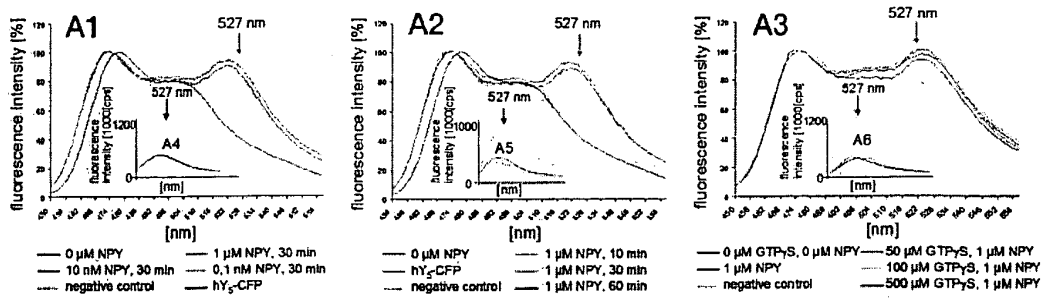
Effect of NPY and GTPγS—In further studies cotransfected cells were incubated with different concentrations of GTPγS in the presence of 1 μM NPY for 30 min. GTPγS is an analog of GTP, and after binding to the G-protein hydrolization is not possible anymore. Therefore, the G-protein stays in the activated form and is no longer accessible for the receptor.

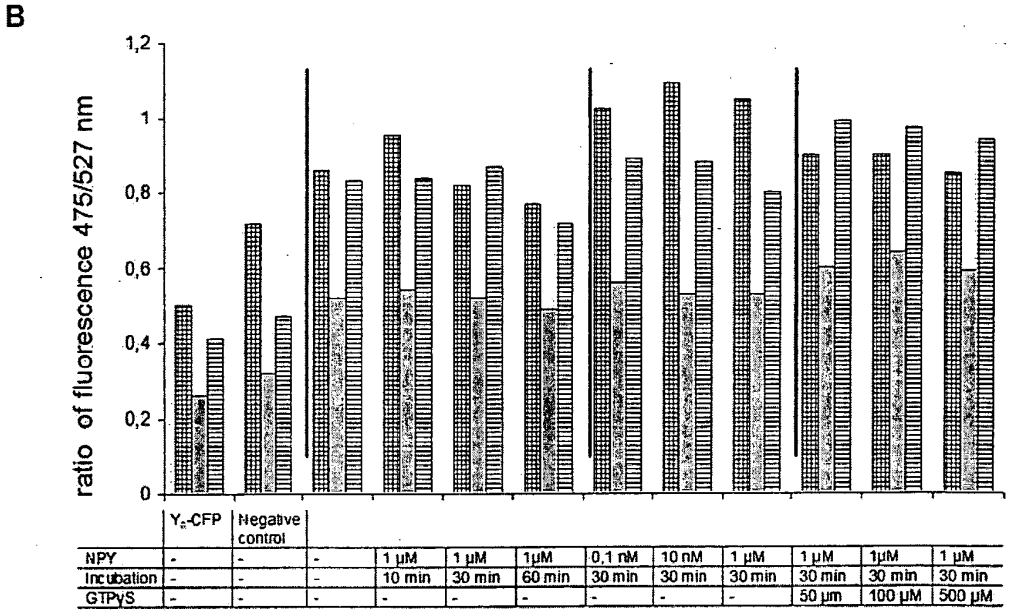
For all three receptor subtypes no significantly different spectra were measured from cells stimulated with different concentrations of GTPγS (Fig. 5, *A3* and *B*). The slight variations are not statistically significant and are rather because of the transiently cotransfected cells.

Receptor Density of Transfected Cells—From investigation with BHK cells stably expressing the hY_2-GFP receptor fusion protein, we know that each cell expresses around 7000 binding sites. Correlation of the extinction coefficient of GFP at 489 nm excitation (ϵ = 55000) and fluorescence intensity at 513 nm allowed us to roughly estimate binding sites in transiently transfected cells. Around 25400 binding sites could be calculated for transiently transfected BHK cells with hY_2-GFP (Table II). Assuming that an average diameter of a BHK cell is 10 μm, and the surface of a GPCR is around 3 nm (helix-diameter, 0.5 nm) the mean distance between two receptors should be ~250 nm in case of equal distribution. This is by far more distant than the half-maximal FRET of 4.7 or 4.9 nm for CFP/YFP or GFP/DSRed pairs, respectively (45).

In parallel it was possible to determine the amount of receptors from cells that were transiently transfected with the CFP and YFP fusion proteins alone or together by using ϵ_{488} (42500 for YFP) and ϵ_{433} (26000 for CFP) as shown in Table II. The total amount of receptors, expressed by cotransfected cells, is lower than 25410 in any case. Cells cotransfected with hY_1-receptor fusion proteins express the lowest amount of receptors. Furthermore, we investigated a lower expression rate for the CFP fusion proteins for cells cotransfected with the hY_1- and hY_5-receptor fusion proteins, respectively, than for the receptors fused to YFP. Cells cotransfected with hY_2-receptor tagged with CFP and YFP express nearly the same amount of both fusion proteins.

Cells were also cotransfected with different amounts of receptor fusion protein DNA (1, 3, 6.5, 13 μg), and the same





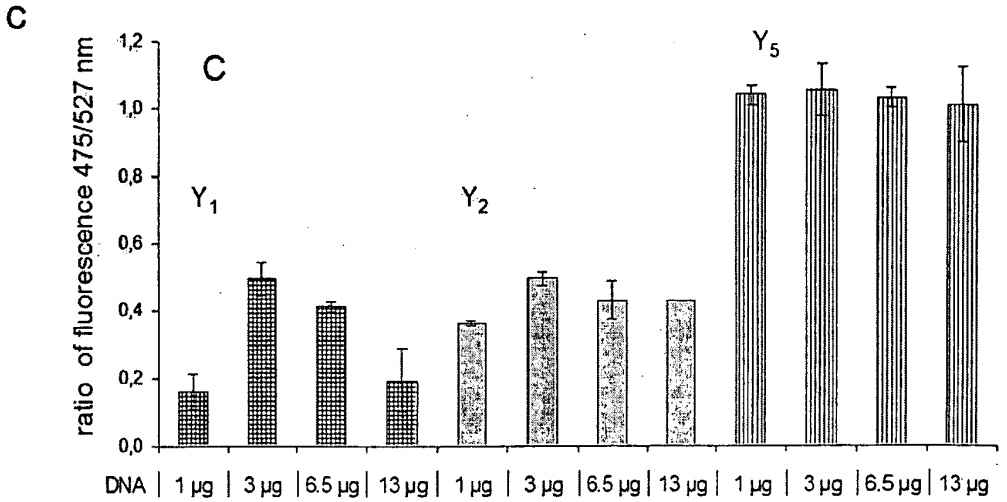


FIG. 5. **FRET studies performed with fluorescence spectroscopy.** BHK cells were transiently cotransfected with the same NPY receptor subtype fused to the FRET pair CFP/YFP. Whole cells were used for measurements with a spectrofluorometer. The emission spectra were recorded with an excitation at 433 nm for FRET and an excitation at 488 nm for normal YFP fluorescence. The detection of the FRET effect is at 527-nm emission. *A*, spectra recorded from cells cotransfected with the hY_5-receptor fused to CFP and YFP by using different conditions. *B*, calculated ratio of the fluorescence intensity at 475/527 nm obtained from recorded fluorescence spectra by using cotransfected BHK cells (*hatched*, hY_1; *solid*, hY_2; *striped*, hY_5). *C*, BHK cells were transiently cotransfected with different amounts of NPY receptor fusion protein DNA, and emission spectra were recorded. The ratio of the fluorescence intensity 475/527 nm is shown (*hatched*, hY_1; *solid*, hY_2; *striped*, hY_5).

amount of each receptor subtype fused to CFP and YFP, respectively, was used. The same fluorescence intensity at 527 nm after excitation with 433 nm was observed independent from the DNA concentration (Fig. 5*C*). Accordingly, the FRET effect is independent from the amount of receptor expression. The very low signals for the hY_1-receptor using 1 and 13 μg of DNA is caused by an extremely low transfection rate found in these cases. In any case, it can be clearly excluded that FRET

TABLE II

Calculated binding sites of transiently transfected BHK cells

From saturation studies of BHK cells stably expressing hY_2-GFP receptors, the amount of binding sites is known. Using the same amount of cells, the given ε_{488} of GFP (55000) and the measured fluorescence intensity at 509-nm emission the calculation of binding sites of transiently transfected cells expressing hY_2-GFP fusion protein is possible. In parallel, because of the measurement of fluorescence intensity, we calculated the receptor density of cells cotransfected with NPY receptors tagged with CFP and YFP, respectively, because the ε_{433} of CFP (26000) and ε_{488} YFP (42500) is also known.

Fusion protein	ε	Receptors/cell
	$cm^{-1}{}_M{}^{-1}$	
hY_2-GFP (stable)	55,000	7063 ± 313
hY_2-GFP (transient)	55,000	25,416 ± 1232
hY_1-CFP	26,000	1793 ± 13
hY_1-YFP	42,500	3365 ± 101
hY_1-CFP/hY_1-YFP	26,000/42,500	2917 ± 49/6378 ± 247
hY_2-CFP	26,000	7806 ± 312
hY_2-YFP	42,500	17,109 ± 547
hY_2-CFP/hY_2-YFP	26,000/42,500	9394 ± 212/10,175 ± 469
hY_5-CFP	26,000	5501 ± 165
hY_5-YFP	42,500	19,743 ± 1955
hY_5-CFP/hY_5-YFP	26,000/42,500	5881 ± 410/10,889 ± 809

occurs only from the close distance because of the overexpression of the receptor fusion proteins.

DISCUSSION

The first speculations on homodimerization of NPY receptors come from studies on the hY_1-receptor antagonist GR231118 (42) and from studies with two peptide ligands, [P_{30},C_{31},F_{32},L_{34}] NPY28–36 and [P_{30},C_{31},W_{32},L_{34}]NPY28–36, selective for the hY_1- and the hY_4-receptors. GR231118, a homodimer of the carboxy-terminal part of NPY, shows a much higher affinity for the hY_1-receptor than the monomer. The same result was found for the two truncated NPY analogues, [P_{30},C_{31},F_{32},L_{34}] NPY28–36 and [P_{30},C_{31},W_{32},L_{34}]NPY28–36, which bind as dimers with a higher affinity to the hY_1-receptor than as monomer. We assumed that homodimerization of the hY_1-receptor could be the reason for the higher affinity. Thus, we decided to investigate homodimerization of the NPY receptor subtypes. Published data demonstrate that FRET is a suitable technique for the investigation of protein-protein interaction (33, 34, 52, 53). Accordingly, NPY receptors tagged with GFP variants, which can be used as FRET pairs, were generated.

After the successful cloning of the receptor fusion proteins mammalian cells were transfected with the cloned fusion proteins, and binding studies, as well as cAMP studies, were performed. These functional studies proved that the receptor fusion proteins are still active and functional. Fluorescent proteins that are expressed in the cytoplasm, in parallel, do not influence the binding behavior of NPY to the hY_1-, hY_2-, and hY_5-receptors. The IC_{50} values calculated from binding studies at the fusion proteins are comparable with the IC_{50} values calculated from binding studies with the wt receptors. The inhibition of cAMP demonstrates that the tagged receptors are still able to bind to G-proteins that inhibit the adenylyl cyclase. The signal transduction process is obviously not disturbed by the fluorescent proteins.

After investigation of cotransfected cells that express hY_1-, hY_2-, and hY_5-FRET pairs, respectively, a FRET effect was observed by using fluorescence microscopy, as well as fluorescence spectroscopy. FRET effect was observed for all three different NPY receptor subtypes, for the hY_1-, hY_2-, and hY_5-receptor. We suggest that these FRET effects are caused by receptor homodimerization or oligomerization, because FRET is only possible when the distance between the FRET pairs is very low. The performed negative controls from fluorescence microscopy and fluorescence spectroscopy confirm that FRET is not an effect of the fluorescence proteins itself. FRET was only obtained for cells containing receptor fusion protein pairs or receptor-tandem constructs produced as positive control.

TABLE III

Ratio of fluorescence intensity at 527 and 475 nm

Spectra were recorded as described under "Experimental Procedures." The ratio between the maxima at 527 nm (FRET) and the maxima at 475 nm (CFP) was calculated. The higher the $ratio_{527/475}$ the higher the effect of FRET. The highest ratio was calculated for the tandem, and the dimerization rate was set to 100%. Correspondingly, the dimerization rate of the cotransfected cells could be calculated.

Fusion protein	Maxima at 527 nm/ maxima at 475 nm	Relative % of receptors as homodimers
hY_2-YFP-CFP	2.14 ± 0.05	100 ± 2
hY_1-CFP/hY_1-YFP	0.94 ± 0.09	44 ± 4
hY_2-CFP/hY_2-YFP	0.56 ± 0.08	26 ± 4
hY_5-CFP/hY_5-YFP	0.87 ± 0.07	41 ± 3

The signal at 527 nm was also not an effect of YFP itself. Spectra recorded with excitation from YFP alone (433-nm) showed a much lower signal at 527-nm emission compared with spectra of cotransfected cells. Furthermore, the FRET fluorescence intensity of the negative control at 527 nm is much lower although the YFP expression itself is much stronger compared with cotransfected cells (Fig. 5, *A4*, *A5*, and *A6*, *green lines versus all other lines*). Accordingly, the maxima at 527 nm cannot be caused by the receptor-YFP fusion proteins.

Furthermore, the existence of FRET between the chosen FRET pairs is confirmed by the generated positive controls containing the FRET pairs in the same molecule. Comparing the results from cells cotransfected with receptor fusion proteins and with the positive control, respectively, identical images from fluorescence microscopy and the identical shape of the fluorescence spectra were obtained. As expected, the emission maxima at 527 nm from the positive control was stronger compared with the signal from cotransfected cells, because every single FRET pair provokes an energy transfer. Subsequently this was taken as the maximal FRET.

The calculation of the receptor density on the cell surface of cotransfected cells and the cotransfection of cells with different DNA concentrations showed that the measured FRET effect is not an effect of receptor overexpression. The average distance between two isolated receptors should be so far that no FRET effect can be recognized.

To investigate a ligand-induced FRET effect cells were cotransfected with each receptor subtype tagged with the FRET pairs, respectively, and incubated with different concentrations of NPY or for different periods. The applied NPY concentrations were chosen in that way, such that different numbers of occupied NPY receptors were obtained. The IC_{50} value of NPY for the used receptors is around 1 nM. Therefore, in case of 1 μM NPY nearly all receptors are occupied, and using 10 and 0.1 nM, accordingly less. The different incubation periods allow the detection of a fast or slow FRET effect. However, all recorded spectra presented no significant difference at 527-nm emission. No increase or decrease of the FRET effect was found for any receptor. For the hY_1-receptor no ligand-induced increase or decrease of FRET was observed from fluorescence spectroscopy, as well as from fluorescence microscopy.

Furthermore cotransfected cells expressing the receptor fusion proteins were incubated with different concentrations of GTPγS in the presence of the same concentration of NPY, respectively. Again for all receptor subtypes no change of the FRET effect was measured. Taken the results from the fluorescence spectroscopy after NPY and NPY/GTPγS stimulation

together we suggest that NPY receptor homodimerization of the investigated receptor subtypes is induced neither by NPY nor by GTPγS. We speculate that the receptors are not transported in the single form to the cell membrane, and therefore they do not form dimers after ligand stimulation. Accordingly, NPY receptors assemble as dimeric units in the endoplasmatic reticulum already, and the receptors are transported as homodimers to the cell membrane.

For the hY_1- and the hY_5-receptors a stronger emission maximum at 527 nm was recorded, which results from FRET. The ratio between the emission maxima at 527 and 475 nm was determined. The maximum at 475 nm was used for the ratio calculation. A high ratio reflects a high dimerization rate or a smaller distance between the fluorescence molecules. Accordingly, we suggest that the dimerization state of hY_1- and hY_5-receptors is higher than that from the hY_2-receptors supposing that the distances between the fusion proteins are the same. This can be assumed because of the same overall organization of the receptor fusion proteins; however, a difference in orientation cannot be fully excluded. Calculation based on 100% FRET of the tandem suggests 41–44% receptor dimers for Y_1 and Y_5 *versus* 26% for the Y_2-receptor (Table III) whereas it has to be kept in mind that percentages might be influenced, as well, by a different geometry of tandem and dimer, as well as by CFP-CFP and YFP-YFP dimers. However, comparing the different receptor subtypes, it became clear that approximately half as many Y_2-receptors are present in dimers compared with Y_1- and Y_5-receptors. The same result was obtained from fluorescence microscopy by pixel-to-pixel analysis and photobleaching in case of the Y_5-receptor. Although the expression rate of the hY_1-receptor is the lowest of the investigated receptor subtypes, the dimerization state is higher than that of the hY_2-receptor. Furthermore, cells cotransfected with hY_2-receptor FRET pair express the highest amount of hY_2-CFP fusion protein compared with the other two receptor subtypes. However, this higher amount of the FRET donor does not result in an increased FRET effect. Accordingly, the number of receptors does not influence the dimerization state, but the dimerization state is dependent on the receptor subtype. As the hY_1- and the hY_5-receptors are involved in some identical physiological roles like regulation of food intake dimerization can be speculated to be correlated with function. Furthermore, subtype-specific dimerization could lead to subtype-specific receptor internalization or recycling, when more than one receptor is expressed on cells.

Galzi and co-workers (54) also generated an NPY hY_1-receptor fusion protein with GFP. In this case the hY_1-receptor was labeled at the amino terminus and not at the carboxyl terminus. This fusion protein was successfully applied for the investigation of receptor internalization and for measurements with a spectrofluorometer. These data confirm that NPY receptor fusion proteins with GFP are useful tools for the investigation of NPY receptors.

In summary our data show for the first time that NPY receptors fused with GFP or GFP variants at the carboxy-terminal part are still fully functional. They are able to bind the natural ligand and able to activate second messengers.

In addition, the establishment and characterization of a cell line that stably expresses the hY_2-GFP fusion protein was successful. This will be a useful tool to further characterize receptor internalization and production.

Furthermore, the fusion proteins were successfully applied for the investigation of receptor interaction. We used two different FRET pairs and different FRET techniques for these investigations, which led to the same results. After cotransfection of cells with the hY_1-, hY_2-, and hY_5-receptor subtypes fused to the FRET pairs, respectively, FRET was observed for each NPY receptor subtype, and we suggest that the FRET effect is caused by receptor dimerization. FRET was not induced by NPY, and also stimulation with GTPγS/NPY did not influence FRET.

In conclusion, we suggest that the hY_1-, hY_2-, and hY_5-NPY receptors subtypes are able to form homodimers and that the homodimerization is induced neither by the ligand nor by GTPγS. Further studies on receptor heterodimerization are currently in progress.

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REFERENCES

1. Beck-Sickinger, A. G. (1996) *Drug Discov. Today* **1,** 502–513
2. Edwards, S. W., Tan, C. M., and Limbird, L. E. (2000) *Trends Pharmacol. Sci.* **21,** 304–308
3. Sautel, M., and Milligan, G. (2000) *Curr. Med. Chem.* **7,** 889–896
4. Devi, L. A. (2001) *Trends Pharmacol. Sci.* **22,** 532–537
5. Dean, M. K., Higgs, C., Smith, R. E., Bywater, R. P., Snell, C. R., Scott, P. D., Upton, G. J., Howe, T. J., and Reynolds, C. A. (2001) *J. Med. Chem.* **44,** 4595–4614
6. Gomes, I., Jordan, B. A., Gupta, A., Trapaidze, N., Nagy, V., and Devi, L. A. (2000) *J. Neurosci.* **20,** RC110
7. Overton, M. C., and Blumer, K. J. (2000) *Curr. Biol.* **10,** 341–344
8. Maggio, R., Barbier, P., Colelli, A., Salvadori, F., Demontis, G., and Corsini, G. U. (1999) *J. Pharmacol. Exp. Ther.* **291,** 251–257
9. Angers, S., Salahpour, A., and Bouvier, M. (2002) *Annu. Rev. Pharmacol. Toxicol.* **42,** 409–435
10. Hebert, T. E., Moffett, S., Morello, J. P., Loisel, T. P., Bichet, D. G., Barret, C., and Bouvier, M. (1996) *J. Biol. Chem.* **271,** 16384–16392
11. Angers, S., Salahpour, A., Joly, E., Hilairet, S., Chelsky, D., Dennis, M., and Bouvier, M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 3684–3689
12. Jordan, B. A., Trapaidze, N., Gomes, I., Nivarthi, R., and Devi, L. A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 343–348
13. Cvejic, S., and Devi, L. A. (1997) *J. Biol. Chem.* **272,** 26959–26964
14. Robbins, M. J., Ciruela, F., Rhodes, A., and McIlhinney, R. A. (1999) *J. Neurochem.* **72,** 2539–2547
15. Bai, M., Trivedi, S., and Brown, E. M. (1998) *J. Biol. Chem.* **273,** 23605–23610
16. Pace, A. J., Gama, L., and Breitwieser, G. E. (1999) *J. Biol. Chem.* **274,** 11629–11634
17. Zhang, Z., Sun, S., Quinn, S. J., Brown, E. M., and Bai, M. (2001) *J. Biol. Chem.* **276,** 5316–5322
18. Zeng, F. Y., and Wess, J. (1999) *J. Biol. Chem.* **274,** 19487–19497
19. Maggio, R., Vogel, Z., and Wess, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 3103–3107
20. Zhu, X., and Wess, J. (1998) *Biochemistry* **37,** 15773–15784
21. Rocheville, M., Lange, D. C., Kumar, U., Sasi, R., Patel, R. C., and Patel, Y. C. (2000) *J. Biol. Chem.* **275,** 7862–7869
22. Pfeiffer, M., Koch, T., Schroder, H., Klutzny, M., Kirscht, S., Kreienkamp, H. J., Hollt, V., and Schulz, S. (2001) *J. Biol. Chem.* **276,** 14027–14036
23. Nimchinsky, E. A., Hof, P. R., Janssen, W. G., Morrison, J. H., and Schmauss, C. (1997) *J. Biol. Chem.* **272,** 29229–29237
24. Zawarynski, P., Tallerico, T., Seeman, P., Lee, S. P., O'Dowd, B. F., and George, S. R. (1998) *FEBS Lett.* **441,** 383–386
25. Gines, S., Hillion, J., Torvinen, M., Le Crom, S., Casado, V., Canela, E. I., Rondin, S., Lew, J. Y., Watson, S., Zoli, M., Agnati, L. F., Verniera, P., Lluis, C., Ferre, S., Fuxe, K., and Franco, R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 8606–8611
26. Jordan, B. A., and Devi, L. A. (1999) *Nature* **399,** 697–700
27. Ng, G. Y., O'Dowd, B. F., Lee, S. P., Chung, H. T., Brann, M. R., Seeman, P., and George, S. R. (1996) *Biochem. Biophys. Res. Commun.* **227,** 200–204
28. Abe, J., Suzuki, H., Notoya, M., Yamamoto, T., and Hirose, S. (1999) *J. Biol. Chem.* **274,** 19957–19964
29. Rodriguez-Frade, J. M., Vila-Coro, A. J., de Ana, A. M., Albar, J. P., Martinez, A. C., and Mellado, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3628–3633
30. Selvin, P. R. (2000) *Nat. Struct. Biol.* **7,** 730–734
31. Cornea, A., Janovick, J. A., Maya-Nunez, G., and Conn, P. M. (2001) *J. Biol. Chem.* **276,** 2153–2158
32. Selvin, P. R. (1995) *Methods Enzymol.* **246,** 300–334
33. Schmid, J. A., Scholze, P., Kudlacek, O., Freissmuth, M., Singer, E. A., and Sitte, H. H. (2001) *J. Biol. Chem.* **276,** 3805–3810
34. Sorkin, A., McClure, M., Huang, F., and Carter, R. (2000) *Curr. Biol.* **10,** 1395–1398
35. Michel, M. C., Beck-Sickinger, A., Cox, H., Doods, H. N., Herzog, H., Larhammar, D., Quirion, R., Schwartz, T., and Westfall, T. (1998) *Pharmacol. Rev.* **50,** 143–150
36. Blomqvist, A. G., and Herzog, H. (1997) *Trends Neurosci.* **20,** 294–298
37. Cabrele, C., and Beck-Sickinger, A. (2000) *J. Peptide Sci.* **6,** 97–122
38. Wahlestedt, C., Yanaihara, N., and Hakanson, R. (1986) *Regul. Pept.* **13,** 307–318
39. Flood, J. F., Hernandez, E. N., and Morley, J. E. (1987) *Brain Res.* **421,** 280–290
40. Inui, A. (1999) *Trends Pharmacol. Sci.* **20,** 43–46
41. Calza, L., Giardino, L., Zanni, M., Velardo, A., Parchi, P., and Marrama, P. (1990) *Regul. Pept.* **27,** 127–137
42. Matthews, J. E., Jansen, M., Lyerly, D., Cox, R., Chen, W. J., Koller, K. J., and

Daniels, A. J. (1997) *Regul. Pept.* **72,** 113–119
43. Daniels, A. J., Matthews, J. E., Slepetis, R. J., Jansen, M., Viveros, O. H., Tadepalli, A., Harrington, W., Heyer, D., Landavazo, A., Leban, J. J., *et al.* (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 9067–9071
44. Harpur, A. G., Wouters, F. S., and Bastiaens, P. I. (2001) *Nat. Biotechnol.* **19,** 167–169
45. Patterson, G. H., Piston, D. W., and Barisas, B. G. (2000) *Anal. Biochem.* **284,** 438–440
46. Chan, F. K., Siegel, R. M., Zacharias, D., Swofford, R., Holmes, K. L., Tsien, R. Y., and Lenardo, M. J. (2001) *Cytometry* **44,** 361–368
47. Pollok, B. A., and Heim, R. (1999) *Trends Cell Biol.* **9,** 57–60
48. Morgenstern, J. P., and Land, H. (1990) *Nucleic Acids Res.* **18,** 1068
49. Dinger, M. C., and Beck-Sickinger, A. G. (2002) *Mol. Biotechnol.* **21,** 9–18
50. Gordon, G. W., Berry, G., Liang, X. H., Levine, B., and Herman, B. (1998) *Biophys. J.* **74,** 2702–2713
51. Jiang, X., and Sorkin, A. (2002) *Mol. Biol. Cell* **13,** 1522–1535
52. Miyawaki, A., and Tsien, R. Y. (2000) *Methods Enzymol.* **327,** 472–500
53. Mitra, R. D., Silva, C. M., and Youvan, D. C. (1996) *Gene* **173,** 13–17
54. Gicquiaux, H., Lecat, S., Gaire, M., Dieterlen, A., Mely, Y., Takeda, K., Bucher, B., and Galzi, J. L. (2002) *J. Biol. Chem.* **277,** 6645–6655